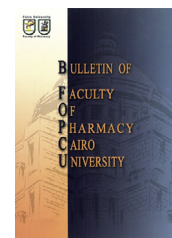




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**ORIGINAL ARTICLE**

# HPLC method validation for modernization of the tetracycline hydrochloride capsule USP monograph



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Received 3 May 2014; accepted 24 August 2014

Available online 26 September 2014

**KEYWORDS**

Tetracycline hydrochloride capsule;  
Impurities;  
US Pharmacopeia monograph;  
Reversed-phase HPLC;  
Method validation

**Abstract** This paper is a continuation to our previous work aiming at development and validation of a reversed-phase HPLC for modernization of tetracycline-related USP monographs and the USP general chapter <226>. Previous results showed that the method is accurate and precise for the assay of tetracycline hydrochloride and the limit of 4-epianhydrotetracycline impurity in the drug substance and oral suspension monographs. The aim of the current paper is to examine the feasibility of the method for modernization of USP tetracycline hydrochloride capsule monograph. Specificity, linearity, accuracy and precision were examined for tetracycline hydrochloride assay and 4-epianhydrotetracycline limit. The method was linear in the concentration range from 80% to 160% ( $r > 0.9998$ ) of the assay concentration (0.1 mg/mL) for tetracycline hydrochloride and from 50% to 150% ( $r > 0.997$ ) of the acceptance criteria specified in tetracycline hydrochloride capsule monograph for 4-epianhydrotetracycline (NMT 3.0%). The recovery at three concentration levels for tetracycline hydrochloride assay was between 99% and 101% and the RSD from six preparations at the concentration 0.1 mg/mL is less than 0.6%. The recovery for 4-epianhydrotetracycline limit procedure over the concentration range from 50% to 150% is between 96% and 102% with RSD less than 5%. The results met the specified acceptance criteria.

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**1. Introduction**

At present, the United State Pharmacopeia (USP) strives to keep its monographs current with advances in technology.

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Peer review under responsibility of Faculty of Pharmacy, Cairo University.

The USP dedicated several monographs for the determination of tetracycline hydrochloride and 4-epianhydrotetracycline hydrochloride impurity in different dosage forms using an HPLC method.<sup>1</sup> Some of these monographs refer to the method outlined in the general chapter <226> for the limit of 4-epianhydrotetracycline hydrochloride. This method uses open column technology which is very old and is a target of modernization by the USP. The HPLC assay method used in the aforementioned monographs is tedious, uses undesirable reagent (dimethyl formamide) and, hence, needs modernization.

As a result, we have developed a simple and robust HPLC method for simultaneous determination of tetracycline hydrochloride and 4-epianhydrotetracycline hydrochloride.<sup>2</sup> This method will replace both the present HPLC method in tetracycline-related monographs and the open column method prescribed in the general chapter <226>, with the intent of referencing all relevant monographs to the general chapter. The new method proved specific, linear, accurate and precise for the drug substance and oral suspension. The advantage of this method over other HPLC methods<sup>3–13</sup> and other electrophoretic methods<sup>14,15</sup> is that it is simple, specific and uses advanced instrumentations which are available in all laboratories for routine analysis. Thus, it can be recommended as a compendium method.

Herein, we examine the feasibility of our previously reported reversed-phase HPLC method<sup>2</sup> for the determination of tetracycline hydrochloride and the limit of 4-epianhydrotetracycline hydrochloride impurity in capsules. The specificity, linearity, accuracy and precision of the method for both compounds are adequately discussed. This work aims at modernization of tetracycline hydrochloride capsule USP monograph.

## 2. Materials and methods

### 2.1. Materials

Tetracycline hydrochloride (97.6% purity, Lot L1H374), epitetracycline hydrochloride (Lot G0E261), and 4-epianhydrotetracycline hydrochloride (Lot K0F299) were USP Reference Standards (Rockville, MD, USA). Anhydrotetracycline hydrochloride (Lot SZBA299XV) was purchased from Sigma-Aldrich (Louis, MO, USA). Tetracycline hydrochloride capsule (250 mg) was obtained from TEVA Pharmaceutical (Rockville, MD, USA).

All reagents were of analytical grade. Water was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA).

### 2.2. Chromatographic conditions

The HPLC system was Waters 2695 Alliance systems controlled with Waters Empower 2 software. We followed the same chromatographic conditions described previously.<sup>2</sup> We used a Phenomenex (Torrance, CA, USA) Prodigy ODS-3, 3  $\mu$ m, 4.6  $\times$  150 mm column. The column temperature was set at 50 °C. The autosampler temperature was kept at 4 °C. Eluent components were 0.1% H<sub>3</sub>PO<sub>4</sub> and acetonitrile. The proportion of acetonitrile was varied from 15% to 40% in 7.5 min, back to 15% in 0.1 min, and held at 15% for 2.4 min. The flow rate was 1.0 mL/min. The detection wavelength was 280 nm. The injection volume was 10  $\mu$ L.

### 2.3. Diluent

The diluent was prepared by mixing 2 mL of 85% phosphoric acid with 2 L of water.

### 2.4. System suitability solution

We used a system suitability solution containing 25  $\mu$ g/mL of each of the following compounds: tetracycline hydrochloride,

epitetracycline hydrochloride, 4-epianhydrotetracycline hydrochloride, and anhydrotetracycline hydrochloride.<sup>2</sup>

### 2.5. Standard solutions

A standard solution of tetracycline hydrochloride (0.1 mg/mL) was prepared in a 100 mL measuring flask by dissolving an accurately weighed 10 mg of USP tetracycline hydrochloride RS in 50 mL diluent and completing the flask to mark with the diluent. The standard solution for the limit procedure had a concentration corresponding to that for the limit procedure stated in the USP-relevant monograph. The limit for tetracycline hydrochloride capsule monograph is 3.0%, so the standard solution concentration was 3.0  $\mu$ g/mL of 4-epianhydrotetracycline hydrochloride.

### 2.6. Validation solutions for the assay procedure

#### 2.6.1. Accuracy stock solution (0.1 mg/mL tetracycline hydrochloride)

An accurately weighed 20 capsules were emptied into a mortar. The capsule shells were weighed and the net content was calculated. The capsule contents were well mixed using a pestle. An accuracy stock solution was prepared in a 1000 mL volumetric flask by dissolving an accurately weighed amount of the mixed powder (equivalent to 100 mg tetracycline HCl) in 500 mL diluent with aid of sonication for 5 min. The flask was then allowed to cool and completed to volume with the diluent.

#### 2.6.2. Linearity solutions

A 160% solution was prepared by dissolving 6 mg of USP tetracycline hydrochloride reference standard in 100.0 mL of the accuracy stock solution. Four other solutions at the concentration levels 80%, 96%, 112%, 144%, were prepared from the 160% solution by diluting 5 mL to 10 mL, 6 mL to 10 mL, 7 mL to 10 mL and 9 mL to 10 mL with the diluent.

#### 2.6.3. Accuracy working solutions

The accuracy solutions were prepared at three concentration levels 120%, 140% and 160%, in triplicate at each level, by spiking the accuracy stock solution with USP tetracycline hydrochloride reference standard according to the following scheme:

- **120% Level:** An accurately weighed 2 mg of USP tetracycline hydrochloride RS was dissolved and diluted to 100.0 mL with the accuracy stock solution.
- **140% Level:** An accurately weighed 4 mg of USP tetracycline hydrochloride RS was dissolved and diluted to 100.0 mL with the accuracy stock solution.
- **160% Level:** An accurately weighed 6 mg of USP tetracycline hydrochloride RS was dissolved and diluted to 100.0 mL with the accuracy stock solution.

#### 2.6.4. Precision solutions

The precision solution was prepared in a 250 mL volumetric flask by dissolving an accurately weighed amount of the mixed powder of capsules equivalent to 25 mg of tetracycline hydrochloride in a 100 mL diluent. The solution was sonicated for 5 min. The flask was allowed to cool and completed to mark with the diluent. Six individual solutions of the same concentration were prepared.

### 2.7. Validation solutions for the limit procedure

The amount of 4-epianhydrotetracycline hydrochloride impurity in tetracycline hydrochloride capsules was estimated using our previously published method<sup>2</sup> to be 0.12%. This amount represents 4% of the maximum limit allowed by the USP for 4-epianhydrotetracycline hydrochloride. Therefore it is not negligible and has been considered during the preparation of the validation solutions.

#### 2.7.1. Solution A

Sample solution (1.0 mg/mL tetracycline hydrochloride). An accurately weighed amount of the mixed powder (equivalent to 100 mg tetracycline HCl) was transferred into a 100.0 mL volumetric flask and dissolved in 50 mL diluent with aid of sonication for 5 min. The flask was allowed to cool and then completed to volume with the diluent.

#### 2.7.2. Solution B

Stock impurity solution. An accurately weighed 7.2 mg of USP 4-epianhydrotetracycline RS, in triplicate, was transferred into a 100 mL volumetric flask, dissolved in 50 mL of the diluent; the flask was then completed to mark with the diluent. Three individual solutions were prepared by diluting 10.0 mL of each solution to 100 mL with diluent.

#### 2.7.3. Linearity, accuracy and precision solutions

These solutions were prepared, in triplicate, at the concentration levels 50%, 75%, 100%, 125% and 150% of the concentration 3 µg/mL of 4-epianhydrotetracycline HCl by spiking the sample solution (solution A) with the stock impurity solutions (solution B) as follows:

- **Standard impurity solution:** Dilute 10.0 mL of solution B to 25.0 mL with diluent.
- **Spike 150%:** Dilute 6.0 mL of solution B and 1.0 mL of solution A to 10.0 mL with diluent.
- **Spike 125%:** Dilute 5.0 mL of solution B and 1.0 mL of solution A to 10.0 mL with diluent.
- **Spike 100%:** Dilute 10.0 mL of solution B and 2.5 mL of solution A to 25.0 mL with diluent.
- **Spike 75%:** Dilute 15.0 mL of solution B and 5.0 mL of solution A to 50.0 mL with diluent.
- **Spike 50%:** Dilute 10.0 mL of solution B and 5.0 mL of solution A to 50.0 mL with diluent.

## 3. Results and discussion

### 3.1. Method validation

We have developed previously a robust HPLC method for modernization of tetracycline-related USP monographs.<sup>2</sup> In brief, the method allowed separation of tetracycline hydrochloride and its related compounds in less than 8 min using a gradient mode of HPLC and a mobile phase composed of 0.1% phosphoric acid and acetonitrile. The peaks were well resolved. Stress studies showed no interference at the retention time of tetracycline hydrochloride and its related compounds from the possible degradants. The method was linear, accurate

and precise for tetracycline hydrochloride and 4-epianhydrotetracycline hydrochloride in the drug substance and oral suspension monographs. Here, we extend the method validation to the tetracycline hydrochloride capsule USP monograph.

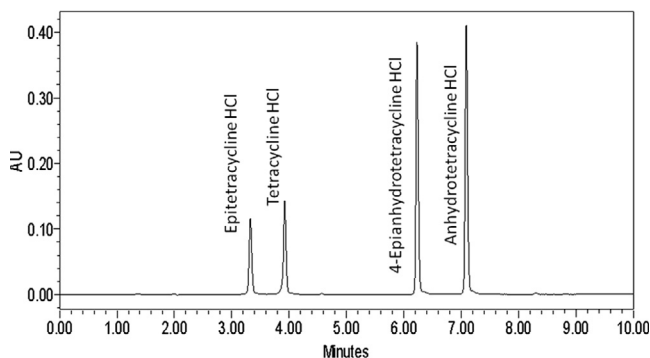
According to USP, system suitability testing is an integral part of analytical methods to evaluate chromatographic parameters of the system.<sup>1</sup> Thus, replicate injections of the system suitability solution and resolution solution were made throughout the validation process. A system suitability chromatogram performed prior to method validation measurements is shown in Fig. 1. The resolution between epitetracycline hydrochloride and tetracycline hydrochloride was always greater than 7 in all system suitability chromatograms, and that between tetracycline hydrochloride and 4-epianhydrotetracycline was greater than 29. The resolution between 4-epianhydrotetracycline and anhydrotetracycline was greater than 11. The resolution was calculated according to the USP guidelines.<sup>1</sup> Tailing for the four peaks was between 0.9 and 1.2 and the precision for the tetracycline hydrochloride and 4-epianhydrotetracycline hydrochloride peaks for five replicate injections was less than 0.5% and 2%, respectively. The retention times were 3.4, 3.9, 6.1 and 7.1 for epitetracycline hydrochloride, tetracycline hydrochloride, 4-epianhydrotetracycline and anhydrotetracycline, respectively. The results indicate that the system suitability requirements are passed and, consequently, further chromatographic parameters can be examined.

### 3.2. Specificity

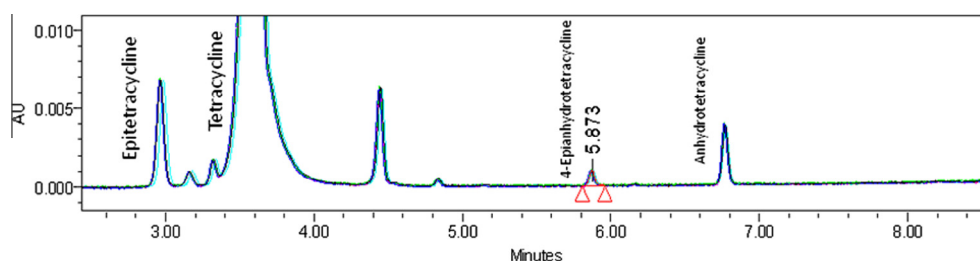
USP defines specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. The specificity of the HPLC method was examined by peak identification; no interferences appeared at the retention time of tetracycline hydrochloride and 4-epianhydrotetracycline hydrochloride in the chromatogram from sample solution of capsule that contains 0.1 mg/mL tetracycline hydrochloride (Fig. 2).

### 3.3. Solution stability

The stability of spiked 100% sample solution was monitored for 24 h. The peak area change for 4-epianhydrotetracycline



**Figure 1** Typical HPLC chromatogram for system suitability solution containing 25 µg/mL each of tetracycline hydrochloride, epitetracycline hydrochloride, 4-epianhydrotetracycline hydrochloride, and anhydrotetracycline hydrochloride.



**Figure 2** Typical chromatogram for capsule solution contains 0.1 mg/mL tetracycline hydrochloride.

hydrochloride was less than 1% for about 6 h. The change increased to about 1.5% after 9 h and 2.5% after 11 h. In contrast, the tetracycline hydrochloride peak was stable for 24 h. The peak area change was less than 0.5%. All subsequent validation measurements were carried out within the time period where the peak area change was less than 1%.

### 3.4. Filter study

A 0.22  $\mu\text{m}$  hydrophilic syringe filter was used for sample solution of 0.1 mg/mL. The filter study showed no effect of the filter on the concentrations of tetracycline hydrochloride and 4-epianhydrotetracycline hydrochloride.

### 3.5. Linearity

Linearity was established by least squares regression analysis of the calibration curve. Calibration curve for the assay method was obtained over the calibration range from 80% to 160% of assay concentration (0.1 mg/mL). The correlation coefficient ( $r$ ) was greater than 0.9998. The results show that an excellent correlation exists between the peak area and concentration of the analyte. The regression equation is as follows:

$$y = 188117x + 80.146 \quad (1)$$

Linear calibration plots for the 4-epianhydrotetracycline hydrochloride limit method was obtained for three sets of concentrations each cover the concentration range from 50 to 150% of the limit concentration 3  $\mu\text{g/mL}$ . The correlation coefficients ( $r$ ) obtained were greater than 0.997. The regression equations for the three sets were as follows:

$$y = 39158x + 9017, r = 0.998 \quad (2)$$

$$y = 42625x + 6214, r = 0.997 \quad (3)$$

$$y = 40984x + 1844, r = 0.999 \quad (4)$$

The results met the specified acceptance criteria for the assay procedure ( $r$  is not less than 0.999) and for the limit procedure ( $r$  is not less than 0.99).

### 3.6. Accuracy and precision of the assay procedure

Instrument precision was determined by replicate injections of the resolution solutions. The relative standard deviation (%RSD) for the tetracycline peak response was below 1.0%. USP precision is defined as the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample, and accuracy is, the closeness of test results to the true value.

**Table 1** Accuracy and precision results for the tetracycline hydrochloride assay procedure.

Level (%)	Spiking conc. (µg/mL)	Conc. found (µg/mL)	Recovery (%)
<i>Accuracy</i>			
120	22.55	22.34	99.6
	20.38	19.91	
	20.78	21.17	
140	38.73	38.76	100.5
	38.68	39.07	
	38.59	38.77	
160	57.71	58.07	100.8
	58.02	58.84	
	60.80	60.94	
<i>Precision</i>			
100	100.69	101.23	100.54
	101.58	102.31	100.72
	99.81	100.34	100.53
	102.84	102.08	99.27
	101.96	102.31	100.34
	102.40	101.99	99.60
	Average =		100.17
	%RSD (n = 6) =		0.59

Acceptance criteria. Accuracy: The average recovery at each level is between 98% and 102%; precision: The RSD of the assay result from the six preparations is NMT 2.0%.

The accuracy of the proposed method was determined by the standard addition method at three different levels corresponding to 120%, 140%, and 160% of the nominal analytical concentration of 0.1 mg/mL. The mean recovery data obtained for each level as well as for all levels combined were within  $\pm 2\%$ . The %RSD for six preparations at the concentration 0.1 mg/mL was less than 1.0%. The accuracy and precision results are summarized in Table 1. The results met the specified acceptance criteria.

### 3.7. Accuracy and precision of the limit procedure

The precision of the instrument was tested by six replicate injections of standard solution of 4-epianhydrotetracycline hydrochloride. The relative standard deviation (%RSD) of the peak area response was  $< 1.5\%$ . The amount of 4-epianhydrotetracycline impurity in tetracycline hydrochloride capsules was estimated to be 0.12%. This amount is not negligible by the USP and was considered during the preparation of the validation solutions. We constructed calibration lines for three sets of spiked solutions in the concentration range from 50%



**Table 2** Accuracy and precision results for 4-epianhydrotetracycline hydrochloride limit procedure.

Level (%)	Spiking conc. (µg/mL)	Conc. found (µg/mL)	Recovery <sup>a</sup> (%)	RSD <sup>b</sup> (%)
<i>1st set</i>				
50	1.44	1.58	97.60	3.5
75	2.16	2.36		
100	2.88	3.16		
125	3.60	3.72		
150	4.32	4.41		
<i>2nd set</i>				
50	1.55	1.67	96.25%	4.1
75	2.33	2.43		
100	3.10	2.99		
125	3.88	3.96		
150	4.65	4.63		
<i>3rd set</i>				
50	1.43	1.54	101.71%	2.2
75	2.14	2.27		
100	2.86	2.90		
125	3.57	3.71		
150	4.28	4.45		

Acceptance criteria: the recovery is between 95% and 105%; the precision (RSD) is NMT 5%.

The results met the specified acceptance criteria.

<sup>a</sup> %Recovery is the slope of the regression line.

<sup>b</sup> %RSD is the standard error of the regression slope.

to 150% of the accepted limit 3 µg/mL. We converted the signals from the spiking assay into the corresponding recovered concentrations. In further diagram, we plotted the recovered concentration against the spiking concentration. By a subsequent regression, the “recovery function” was obtained for each set of measurements.<sup>16</sup> The regression equations for the 3 set are as follows:

$$y = 0.976x + 0.233, \quad r = 0.998 \text{ for the 1st set}$$

$$y = 0.963x + 0.151, \quad r = 0.997 \text{ for the 2nd set}$$

$$y = 1.017x + 0.069, \quad r = 0.999 \text{ for the 3rd set}$$

The slope of the regression line expresses the recovery and the standard error of the regression slope expresses the precision of the analytical method. The spiking concentration, recovery and precision results are summarized in Table 2.

#### 4. Conclusion

In previous work we proposed an HPLC method for modernization of tetracycline-related USP monographs. The method was validated for the tetracycline hydrochloride drug substance and oral suspension. The present work proves that the method is accurate, precise, specific, linear, and sensitive for the assay of tetracycline hydrochloride and the limit of 4-epianhydrotetracycline hydrochloride in capsule. So far, we have presented the validation results related to the modernization of three tetracycline-related USP monographs. The feasibility of using this procedure for modernization other tetracycline-related USP monographs is still underway.

#### 5. Conflict of interest

I certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

#### Acknowledgments

The author is grateful to the US Pharmacopeia (Rockville, MD, USA) for the Visiting Science Program (VSP) for tetracycline-related USP monograph modernization. Valuable advice and support by Drs. Samir Wahab, Shane Tan and Zarima Kassymbek (Separation Science Laboratory, USP, Rockville, USA) are gratefully acknowledged.

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